MODIFICATION OF THE PROCEDURE FOR THE DETERMINATION OF THE ACID AND LIPID SOLUBLE PHOSPHATES, RNA, AND DNA IN MOUSE LIVER TISSUE AS SUGGESTED BY DUDLEY COPPOCK, AND JOHNSON

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CHAPTER I

INTRODUCTION

The purpose of this study was to develop, if possible, more accurate methods for the determination of the acid and lipid soluble phosphated, DNA, and RNA in mouse hepatic tissues, and to validate them on normal mice.

This work grew out of difficulties encountered in the analysis of mouse livers according to the procedure used by Dudley.[1] Special attention was given to the characteristics of the colorimetric methods and reactions and to specific questions of technique. A resultant modification of Dudley's procedure was checked for reproducibility and used for the analysis of normal, age controlled, A/Jax mice.

Historical background. The first form of the procedure now used at Drake was suggested by Schneider in 1945.[2] Through the influence of the work done by Schmidt and Tannhauser, Schneider published a modified version of

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[1] D.S. Dudley, W.H. Coppock, and L.P. Johnson, "DNA, RNA, Lipid Phosphorus, and Acid Soluble Phosphorus In Normal A/Jax Mouse Livers," Iowa Academy of Science, LXVI (1959), 427-429.

[2] Walter C. Schneider, "Phosphorus Compounds In Animal Tissues," The Journal of Biological Chemistry, CLXI (November, 1945), 293-303.

his original procedure in 1946.[1] It called for the extraction of the acid soluble phosphates with cold 10 percent trichloroacetic acid (TCA), the lipid phosphates with room temperature 95 percent ethanol, and the nucleic acids with hot (90° C) 5 percent TCA. The acid and lipid phosphates were quantitatively identified through the method of Lepage and Umbreit, the DNA with diphenylamine reagent, and the RNA with orcinol reagent.[2]

W.H. Coppock, in 1957, determined the quantity of RNA and DNA through the identification of the phosphorus content by the method of Fister.[3] In early 1958 attention shifted to the details of extraction of the acid and lipid phosphates according to Schneider, and analyzing the extracts for total phosphorus according to Fister.

D.S. Dudley began work on the procedure in July, 1958. His preliminary work centered about the quantitive determination of RNA and DNA using the

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[1]Walter C. Schneider, "Phosphorus Compounds In Animal Tissue," The Journal of Biological Chemistry, CLXIV (August, 1946), 748.

[2]Ibid.

[3]Harold J. Fister, "Inorganic Phosphorus Method P-36b.3," Manual of Standardized Procedures for Spectrophotometric Chemistry, (New York: Standard Scientific Supply Corporation, 1950).

reagents suggested by Schneider,[1] but preparing and applying them according to Snell and Snell.[2] He analyzed twelve normal and four abnormal mice.[3]

During the summer of 1959, W. Vasilake, D. Johns, E. Sutters, L. Spriggs, and L. Carlson used Dudley's method for the analysis of abnormal mice. During that period modifications of previous methods were incorporated, one of which was the replacement of round cuvetts with square ones.

The writer did the bukk of his work from September of 1959 to August of 1960. During that period several modifications of the technique were evaluated, some of which were used during the summer of 1960 by the National Science Foundation Research Participation group.[4]

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[1]Schneider, op. cit., (August, 1946), pp. 294-296.

[2]F.D. Snell and C.T. Snell, Colorimetric Methods of Analysis, (Third Ed., Vol. III, New York: D. Van Nostrand Company Inc., 1953), pp. 446-457.

[3]D.S. Dudley, et al., op. cit., pp. 426-231.

[4]The summary of work done at Drake was found in the Mouse Liver Record Books I and II, Drake Chemistry Department, and through personal discussions.

CHAPTER II

METHODS AND MATERIALS

The constituents of hepatic tissues sought in this analysis were differentially extracted through the use of ice cold 10 percent TCA, 95 percent ethanol at room temperature, and hot (90° C) 5 percent TCA, and identified quantitatively through colorimetric techniques. The accuracy of the procedure was dependent on the manipulative and chemical factors of sample extraction and color development.

I. REAGENTS AND SPECIAL EQUIPMENT

Solutions in general. Percentages of reagents used throughout the work were either weight-volume or volume-volume depending on the physical state of the constituents. The explicit quantities of reagents normally used were as discussed in the detailed procedure for the liver analysis.

Detailed preparation of reagents and indicators. Preparation of the DNA standard was started on the same day and at about the same time as the liver extraction to cancel, as much as possible, errors due to decomposition. Fifty mg. of DNA was weighed out to the nearest 0.1 mg.

and dissolved in 3 ml. of 1 normal KOH in a 30 ml. beaker. This was transferred quantitatively to a 25 ml. volumetric flask and diluted to volume with distilled water. Six ml. of this concentrate was transferred by pipette to a 100 ml. volumetric flask. An equal volume of 10 percent TCA was added (to make the final concentration of TCA more nearly 5 percent) and the flask was filled about two-thirds full with 5 percent TCA. The flask was heated in a 90° C water bath for fifteen minutes and the solution was allowed to cool overnight before being diluted to volume with 5 percent TCA.

The RNA standard, prepared at the same time as the DNA standard, was weighed out in the same quantity and with the same accuracy and treated in the same manner as the DNA except that 8 ml. of the concentrate was transferred to the 100 ml. volumetric flask and 8 ml. of the 10 percent TCA was added instead of the 6 ml. used in the DNA.

One liter of standard phosphate solution, containing 0.08 mg. phosphorus per ml., was prepared by dissolving 351 mg. of pure, dry, monopotassium phosphate (KH_2PO_4) in distilled water, adding 10 ml. of 10 normal H_2SO_4, and diluting to volume with distilled water in a liter volumetric flask.

For one mouse analysis (four aliquots per mouse), 51.4 ml of DNA indicator solution was prepared by adding 1.4 ml. of concentrated H_2SO_4 to 50 ml. of 1 percent diphenylamine in glacial acetic acid.

For one mouse analysis (four aliquots per mouse), 50 ml. of RNA indicator was prepared by dissolving 100 mg. orcinol (1,3-dihydroxy-5-methylbenzene) in 50 ml. of 0.005 percent cupric chloride in concentrated HCl.

For one mouse analysis (four aliquots per mouse), 15 ml. of phosphate indicator was prepared by dissolving 2.25 g. of sodium bisulfite in 13 ml. distilled water and then adding 37.5 mg. of 1-amino-2-napthol-4-sulfoinic acid and 75.0 mg. of anhydrous sodium sulfite.

The ammonium molybdate reagent was prepared by dissolving 2.5 g. of dry, reagent grade $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$ in 100 ml. of distilled water.

Stability of reagents. All reagents are indefinitely stable with the exception of the 5 percent TCA (stable for one week at room temperatures[1]), the aminonaptholsulfonic acid reagent (stable for two weeks if stored in the dark[2] but always made fresh in this

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[1]Harold J. Fister, "Inorganic Phosphorus Method P-36a.1," Manual of Standardized Procedures for Spectrophotometric Chemistry, (New York: Standard Scientific Supply Corporation, 1950).

[2]Ibid.

work), the orcinol reagent which discolors in anywhere from a few days to a few weeks (Dudley found that slight discoloration was not quantitatively detrimental to determinations[1]). Ammonium molybdate is stable for a long period but may form a precipitate. If this occurs, the solution should be discarded.

Special equipment. Volumetric pipetts of 1, 2, 3, 5, and 6 ml., Mohr pipetts of 1 and 10 ml., volumetric flasks of 10, 25, 100, and 1000 ml., a Roller-Smith balance and an analytical balance, a Coleman Model 14 spectrophotometer with a PC-4 filter using square cuvetts, and an A.H. Thomas Co. tissue grinder (No. 4288-B, Size A) were used in the work. There were prepared a container to hold the ice bath and samples during the acid soluble phosphate extraction (a 5 inch high glass cylinder fitted at each end with a number 10 rubber stopper, one of which was bored to hold four 4 inch test tubes and one 8 mm. glass tube air vent which was topped with rubber tubing closed with a pinch clamp) and a test tube holder to permit the simultaneous heating and cooling of solutions (a 4 inch square aluminum plate with handle attached and

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[1]Mouse Liver Record Book II, Drake Chemistry Department, p. 74.

bored with seven holes large enough to hold 6 inch test tubes).

II. INVESTIGATIVE PROCEDURES

Extracted volumes. Although known volumes of solvent were used in each extraction, the volume of the decantant recovered had to be determined. That was done by pipetting the decantants of the three solvent fractions (the acid, lipid, and nucleic acid extract fractions) from aliquots of liver analysis into tared test tubes, weighing, determing the density of the fractions by weighing pipetted volumes, and calculating the total recovered volume.

RNA correction factor. To determine the optical density resulting from the reaction of orcinol reagent on DNA, six 3 ml. solutions containing 12 mg. DNA per 100 ml. (General Biochemicals Inc., lot number 8044-C) were prepared along with four 3 ml. solutions containing 12.3 mg. DNA per 100 ml. These were treated as in the normal procedure for a RNA sample and the optical densities were determined. A blank of 5 percent TCA was treated in the same way and at the same time as the samples. To minimize instrument error, the blank zero was set at an optical density of 0.500.

Morrison checked this work using a concentration of 12 mg. DNA per 100 ml. in each of 6 test tubes (3 ml. per test tube).

Effects of light and dark on phosphate color development. Five 1 ml. aliquots of phosphate standard containing 0.08 mg. phosphorus per ml. were transferred to separate 25 ml. volumetric flasks numbered I through V. One-half ml. of 10 normal sulfuric acid was added to solutions II through V. Then 2.5 ml. of the ammonium molybdate reagent and 1 ml. of the aminonaptholsulfonic acid reducing reagent were added to flasks I and II in a darkened room and to solutions III, IV, and V in a room lighted at a normal level with artificial light. All five solutions were then diluted to volume and throughly shaken without changing their light environment.

Solution I was divided into three parts and solutions II through V into two parts each. The first part of solutions I, II, and III was placed in a square cuvett and the optical density determined often over a period of time with the solutions being moved out of the beam of light between determinations.

The second part of solutions I through V was left in the flask and its optical density determined

only two or three times during the developing period.

The third part of solution I was handled in such of a manner that its optical density was determined more often than the second part but less often than the first.

The first parts of solutions IV and V were placed in separate square cuvetts; their optical densities were taken periodically with solution IV being maintained in the instrument's beam of light between readings for the first 24 minutes after the addition of the indicator. After that time the solutions were reversed so that solution V was in the beam of light between readings.

Effects of diphenylamine and orcinol reagents on acid and lipid extracts. One 3 ml. portion of acid and lipid extract from each aliquot of a mouse liver homogenate was treated as a regular DNA extract and a simular portion was treated as a regular RNA extract. Ten percent TCA and 95 percent ethanol were used for blanks. The optical densities were recorded.

Effect of light and dark on DNA and RNA color development. Five test tubes containing 3 ml. of a solution of 12 mg. DNA per 100 ml. were treated with 6 ml. of diphenylamine reagent and heated for three minutes in a boiling water bath. The color of the solution in the first test tube was allowed to develop

approximately five feet away from a sixty watt bulb with no obstructions interfering with the light. Test tubes Two, Three, and Four were placed in slotted wood test tube stands and left on the top of the desk while the color of the solutions developed. Test tube Five was placed in a desk, shielded from all light, during the color development. The optical densities of these solutions were taken periodically. A spectral scan between 480 and 700 mu was taken periodically on solutions One and Five.

Three ml. from each of the four RNA-DNA extracts of mouse number 16 were treated with indicator and heat as usual for DNA determination along with a standard solution, and the color was allowed to develop in test tubes placed in slotted wood test tube stands sitting on top of the desk. The optical densities were determined periodically and the calculated mg. DNA per 100 g. tissue resulting from each optical density determination were compared.

Three ml. from each of the four liver RNA-DNA extracts of mouse number 16 were also treated with indicator and heat as usual for RNA determination along with a standard solution. The optical densities were determined, the solutions allowed to stand for about 20

hours in test tubes in slotted wood stands and the optical densities were remeasured. The two optical density determinations were compared.

Accuracy of DNA-RNA detection and the adherance of these systems to Beer's Law. Two test tubes for each of four concentrations of DNA (12, 10, 8, and 6 mg. DNA per 100 ml.) containing 3 ml of the respective concentrate, were treated as usual with diphenylamine and heat and the optical densities were determined periodically over a 23 hour span. One solution of each concentration was placed in the light for the duration of color development and the other was placed in the dark.

Six test tubes for each of five concentrations of RNA (12, 10, 8, 6, and 4 mg. RNA--General Biochemicals Inc., lot number 8045-C--per 100 ml.) containing 3 ml. of the respective concentrations were treated as usual with orcinol reagent and heat and the optical densities determined.

In each of the above investigations, the blank used was 5 percent TCA.

As the DNA and RNA standards used in actual liver analysis were of 12 mg. per 100 ml. and 8 mg. per 100 ml.

respectively, these concentrations were assumed to be the standards with which the other concentrations were calculated from the optical densities. The calculated concentrations were compared to the theoretical concentrations to determine the accuracy of the detection.

RNA color development with heating time. Three series of five test tubes containing 3 ml. of a solution of 16 mg. RNA per 100 ml. and two series of seven test tubes containing 3 ml. of a solution of 8 mg. RNA per 100 ml. were prepared. The orcinol reagent (6 ml.) and 5 percent TCA (3 ml.) were added as usual and the test tubes were placed in boiling water. One test tube from each of the four series was removed after 8, 10, 12, 14, 20, 30, and 40 minutes of heating, cooled, and the optical density determined.

III. DETAILED PROCEDURE FOR LIVER ANALYSIS

The following detailed procedure was used for the analysis of the mice in this work. It contains essential elements from Schneider and Dudley, and also the useful modifications which have been incorporated since their work.

Preparation of specimen. The mouse was killed by striking on the back of the neck with the edge of a scapel handle. The liver was immediately removed, the gall bladder discarded, and the liver weighed to the nearest mg. on a Roller-Smith balance. The specific gravity was determined by water immersion (distilled water) and the liver was placed in the tissue grinder tube. Water from the specific gravity immersion was added by volumetric pipette to make a near 20 percent homogenate, the actual percentage being calculated. The liver was finely minced by five to ten minutes of grinding at near 0° C with the tissue grinder being powered by an electric motor. Three or four 1 ml. aliquots of the homogenate were transferred by seriological pipette to separate 4 inch test tubes and the various fractions were extracted in the following manner:

Removal of the acid soluble phosphorus compounds. To the 1 ml. of the homogenate was added 2.5 ml. ice cold 10 percent TCA. The test tube was closed with a rubber stopper which had been previously boiled in KOH solution to remove sulfur, and the test tube was shaken vigorously for at least five minutes in an ice bath. The mixture was then centrifuged, the supernatant liquid carefully transferred by pipette to a 10 ml. volumetric flask, and

the extraction was repeated with a second 2.5 ml. portion of ice cold 10 percent TCA. The second supernatant liquid was added to the first and the combination diluted to volume with 10 percent TCA. This extract was analyzed according to procedure I of inorganic phosphate analysis (page 19).

Removal of the lipid soluble phosphorus compounds. To the residue from the acid extract was added 5 ml. of 1:4, water:95 percent ethanol, mixture. The test tube was closed, shaken for at least 15 minutes, centrifuged for another 15 minutes, and the supernatant liquid was carefully transferred by pipette to a 10 ml. volumetric flask. The extraction was repeated with 5 ml. of 95 percent ethanol, the centrifugation not being so long the second time. The two supernatant liquids were combined and diluted to volume with 95 percent ethanol. This extract was analyzed according to procedure I of inorganic phosphate analysis (page 19) with the exception that 2 ml. of the extract were analyzed (and 2 ml. of 95 percent ethanol used as the blank) instead of the 3 ml. extract (and 3 ml. of 10 percent TCA).

Removal of RNA and DNA. To the residue from the lipid extraction was added 2.0 ml. cold 5 percent TCA.

The test tube was closed, shaken for about 5 minutes, centrifuged, and the supernatant liquid transferred by pipette to a 10 ml. volumetric flask. The residue was resuspended in 5 ml. of 5 percent TCA and heated in a water bath at 90° C for 15 minutes, cooled in running water, centrifuged, and the supernatant liquid added to the first. The residue was resuspended again in 2.0 ml. of 5 percent TCA, shaken for five minutes, centrifuged, and separated as before. The combined extracts were diluted to volume to form the nucleic acid fraction for RNA and DNA. This fraction was analyzed in the following was (the residue was discarded):

Analysis of nucleic acid extract for DNA. Three ml. of the extract and 6 ml. of the DNA indicator solution were combined in a 6 inch test tube, thoroughly mixed, then heated in boiling water for three minutes and allowed to stand overnight in the dark. Three ml. of the standard DNA and 3 ml. of 5 percent TCA (for the blank) were similarly treated at the same time. The optical densities were determined at a wave length of 600 mu using square cuvetts. The mg. of DNA per 100 g. tissue was calculated by the formula:

$$\frac{\text{mg. DNA}}{100 \text{ g. tissue}} = \frac{\text{O.D. Samp.}}{\text{O.D. Std.}} \times \frac{12 \text{ mg. DNA}}{100 \text{ ml.}} \times \frac{3 \text{ ml. Std.}}{3 \text{ ml. Samp.}} \times \frac{10 \text{ ml. extract}}{1 \text{ ml. aliquot}} \times \frac{1 \text{ aliquot}}{y \text{ g. tissue}} \times \frac{100 \text{ g. tissue}}{1 \text{ g. tissue}},$$

where $y = (\frac{\%\ \text{homogenate}}{100} \times 0.985)$ g. liver.

The factor of 0.985 was necessary because E. Suters showed that the seriological pipette delivered only 98.5 percent of the weight of the liver it was theoretically supposed to deliver.[1]

Analysis of nucleic acid extract for RNA. Three ml. of the extract, 3 ml. of 5 percent TCA (to dilute the sample and prevent the optical density from being to high), and 6 ml. of the orcinol reagent were mixed in a 6 inch test tube, heated in boiling water for 20 minutes, cooled in running water to room temperature, and the optical density was determined at 620 mu. Three ml. of the standard and 3 ml. of 5 percent TCA (the blank) were treated in the same manner (with the same dilution) at the same time. The mg. of RNA per 100 g. tissue was calculated by the formula:

$$\frac{\text{mg. RNA}}{100 \text{ g. tissue}} = \frac{\text{O.D. Samp.} - (0.00405)\frac{\text{mg. DNA}}{(100 \text{ ml.})}}{\text{O.D. Std.}} \times$$

[1]Mouse Liver Record Book II, p. 111.

$$\frac{16 \text{ mg. RNA}}{100 \text{ ml.}} \times \frac{3 \text{ ml. Std.}}{3 \text{ ml. Samp.}} \times$$

$$\frac{10 \text{ ml. extract}}{1 \text{ ml. aliquot}} \times \frac{1 \text{ aliquot}}{y \text{ g. liver}} \times$$

$$\frac{100 \text{ g. tissue}}{1 \text{ g. tissue}},$$

where y is the same as in the DNA calculations and the factor subtracted from the optical density of the sample is necessary because DNA gave a color with orcinol reagent. The mg. of DNA per 100 ml. was calculated by the formula:

$$\frac{\text{mg. DNA}}{100 \text{ ml.}} = \frac{\text{O.D. DNA Samp.}}{\text{O.D. DNA Std.}} \times 12 \text{ mg. DNA.}$$

Analysis of acid soluble fraction, procedure I. Three ml. of the acid extract were evaporated to one-half ml. in a 6 inch test tube. The tube was cooled, one-half ml. of 10 normal H_2SO_4 was added, and the evaporation was continued over a hot flame until either SO_3 fumes were evolved or until the sulfuric acid (carrying the coal-black residue with it) began to distil in the tube barrel. The flame was turned down and the tube allowed to cool for five minutes. Ten drops of 30 percent H_2O_2 were added, the fire again turned up, and after the evolution of gas had ceased (about five minutes) the peroxide treatment was repeated. This process was

continued until no further darkening or discoloration was detected after 15 minutes of digestion.

After digestion was complete the tube was allowed to cool, 2 or 3 ml. of distilled water was added, and then the tube was placed in boiling water for about 5 minutes. The solution was transferred quantitatively to a 25 ml. volumetric flask, 2.5 ml. ammonium molybdate reagent was added, and 1 ml. of the aminonaptholsulfonic acid reagent was added. The time of the addition of the latter was recorded. The solution was diluted to volume, shaken, and the color allowed to develop in the flask, on the desk top, out of direct sunlight, for exactly five minutes, each sample being treated separately in this latter step.

The blank (3 ml. of 10 percent TCA treated at the same time and in the same manner as the 3 ml. sample) was the first to be treated with the indicator and, after 5 minutes, its optical density was set at 0.000. The standard (1 ml. of the standard phosphate solution which had been treated at the same time and in the same way as the sample) was the second. All subsequent determinations were made in order, but the zero was never changed despite the fact that the optical density of the blank was changing.

The mg. of phosphorus per 100 g. tissue was calculated by the formula:

$$\frac{\text{mg. P}}{100\text{ g. tissue}} = \frac{\text{O.D. Samp.}}{\text{O.D. Std.}} \times \frac{8\text{ mg. P}}{100\text{ ml.}} \times \frac{1\text{ ml. Std.}}{3\text{ ml. Samp.}} \times \frac{10\text{ ml. extract}}{1\text{ ml. aliquot}} \times \frac{1\text{ aliquot}}{y\text{ g. tissue}} \times \frac{100\text{ g. tissue}}{1\text{ g. tissue}},$$

where y is the same value as before.

The mg. of phosphorus per 100 g. tissue for the lipid extract (again using 1 ml. of the standard phosphate solution as the standard) was calculated by the formula:

$$\frac{\text{mg. P}}{100\text{ g. tissue}} = \frac{\text{O.D. Samp.}}{\text{O.D. Std.}} \times \frac{8\text{ mg. P}}{100\text{ ml.}} \times \frac{1\text{ ml. Std.}}{2\text{ ml. Samp.}} \times \frac{10\text{ ml. extract}}{1\text{ ml. aliquot}} \times \frac{1\text{ aliquot}}{y\text{ g. tissue}} \times \frac{100\text{ g. tissue}}{1\text{ g. tissue}},$$

where y is the same value as before.

Analysis of the acid soluble fraction, procedure II. This analysis procedure was carried out by the summer group (1960) and can be found in detail in their literature. A study of the results of phosphate determinations using both methods was made.

Checking the reproducibility of the procedure. Seven 8 to 9 week old, strain A, Cumberland, female, normal mice were run according to the procedure. Two mice were found to have mottled livers. The results were tabulated and compared.

Variation of constituents with age. The quantity of acid and lipid soluble phosphates, RNA, and DNA were determined with a limited number of normal, A/Jax mice of various ages using the preceeding procedure.

CHAPTER III

RESULTS

The data which were collected by using the methods described in detail in the preceeding discussions have been summarized in this chapter.

Extracted volumes. The volume of the supernatant liquid recovered to form each of the three extract fractions (acid soluble phosphates, lipid soluble phosphates, and nucleic acids) was not equal to the total volume of the solvent used. The data are presented in Table I.

TABLE I

ACTUAL VOLUME IN Ml. OF DECANTANT OBTAINED FROM EXTRACTIONS

Extract Fraction	Mouse Number	Aliquot Number 1	2	3	4
Acid	3	5.51	5.52	5.43	5.46
	6	5.58	5.57	5.53	5.74
	7	5.64	5.43	5.50	5.47
Lipid	3	9.05	8.75	9.06	9.06
	6	9.58	9.59	9.49	9.55
	7	10.0	9.15	9.01	9.14
Nucleic Acid	3	10.8	10.6	10.2	11.1
	6	10.4	10.2	9.88	10.3
	7	10.5	10.7	11.0	10.4

(data to 3 significant figures only)

RNA correction factor. DNA was found to give an average optical density of 0.00405 per mg. with the orcinol reagent. Therefore a factor must be subtracted from the optical density of the RNA to account for the increased optical density due to the presence of DNA in the sample. The data are presented in Table II.

TABLE II

OPTICAL DENSITY OF DNA-ORCINOL REACTION

blank zero set at 0.500 = 620 mu

Mg. DNA / 100 ml.	Sample Number 1	2	3	4	5	6	Avg. O.D.	O.D. / Mg. DNA
24	0.594	0.595	0.597	0.595	0.594	0.595	0.095	0.00399
12.6	0.550	0.550	0.550	0.550	0.550		0.050	0.00405
12.0	0.550	0.549	0.550	0.549	0.549	0.550	0.050	0.00416
							Avg. Optical Density increase/mg. DNA	0.00405

Effects of light and dark on phosphate color development. Development of the molybdenum blue in the phosphate determination was effected by light and time in such of a way that, when the color was developed in the light, the optical density increased much more rapidly than when it was developed in the dark, and when developed over a long period of time there was no suggestion of leveling off until 300 to 350 minutes after

the addition of the indicator. The optical density of solutions developing in large volumes increased less rapidly than that of smaller volumes (i.e. in the flasks and in the cuvetts). The data are presented in Table III and Figure I.

Effects of diphenylamine and orcinol reagents on acid and lipid extracts. The diphenylamine reagent gave no noticeable color formation with either acid or lipid extracts. The orcinol reagent, however, produced a decidedly colored solution when applied to both extracts. The data are presented in Table IV.

TABLE IV

OPTICAL DENSITIES OF ACID AND LIPID EXTRACTS BY DIPHENYLAMINE AND ORCINOL REAGENTS

Reagent	Acid extract sample number					Lipid extract sample number				
	1	2	3	4	Std	1	2	3	4	Std
Diphenylamine	no reaction					no reaction				
Orcinol	.319	.315	.307	.309	.448	.186	.177	.175	.177	.424
Mg. RNA/0.1 l. indicated	11.4	11.2	10.9	11.0		7.01	6.68	6.61	6.68	

Effect of light and dark on DNA and RNA color development. The color development of the DNA samples was continueous with no indication of leveling off. However the rate of the color development was greatly

TABLE III

OPTICAL DENSITY DEVELOPMENT OF MOLYBDENUM BLUE IN LIGHT AND DARK OVER A PERIOD OF TIME

Sln No.	Part No	Develop IN Light	Develop IN Dark	OPTICAL DENSITY																																				
1	1		X				.043	.048	.051	.056	.061	.068	.072	.078	.084	.090	.097	.104	.109	.110	—	.152	.175		.200		.290	.430		.570	.549	.549	.549								
1	2		X																										.277		.360		.461								
1	3		X				.045			.057	.059		.068				.089				.108			.137			.235	.340	.505												
2	1		X	.469	.470	.474	.479	.479	.485	.490	.495	.500	.504	.510	.516	.521	.529	.535	.542	.550	.568	.585	.605		.635		.711	.967	1.98												
2	2		X																							.543	.560		.785												
3	1	X		.473	.475	.480	.484	.489	.490		.497		.519			.525				.558				.649			.900	1.25													
3	2	X										.491														.552	.610	.715													
4	1	IN LIGHT UP TO 23 Min	DARK	.501	.504	.509	.512	.517	.526	.543					.559																											
4	2	X														.549	.552	.562	.568																							
5	1	IN LIGHT UP TO 23 Min		.521	.522	.526	.530	.531	.534	.535					.575																											
5	2	X														.538	.540	.548	.554																							
TIME SINCE START (minutes)				3	4	7	10	13	16	19	22	25	28	31	34	37	40	43	46	49	55	61	67	70	76	79	100	150	200	250	300	350	400	528							

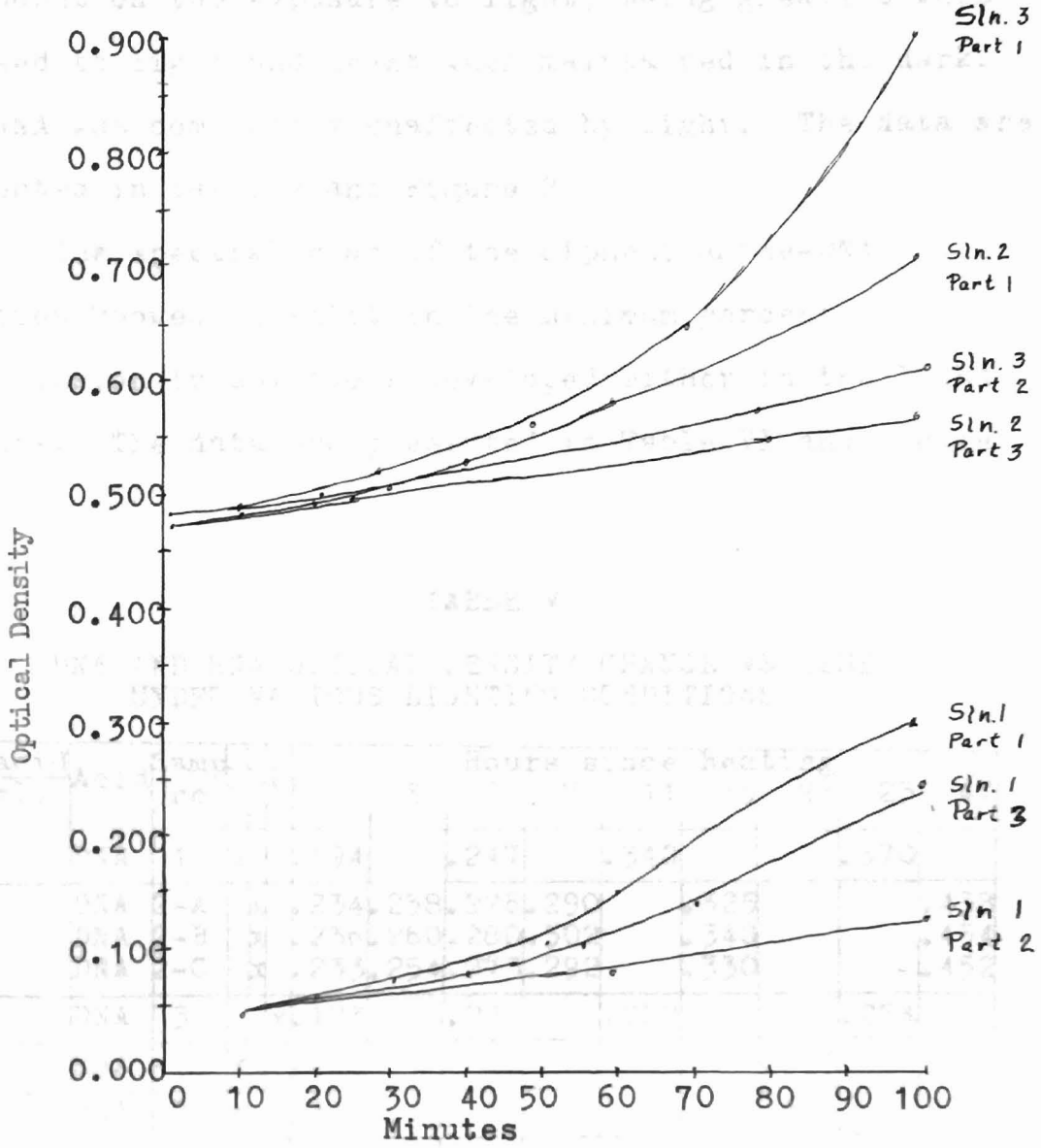

FIGURE 1

MOLBDENUM BLUE DEVELOPMENT IN LIGHT AND DARK: OPTICAL DENSITY VS TIME

dependent on the exposure to light, being greatest when exposed to light and least when maintained in the dark. The RNA was completely unaffected by light. The data are presented in Table V and Figure 2.

The spectral scan of the diphenylamine-DNA reaction showed no shift in the minimum percent transmission in solutions developed either in the light or dark. The data are presented in Table VI and Figure 3.

TABLE V

DNA AND RNA OPTICAL DENSITY CHANGE VS TIME UNDER VARIOUS LIGHTING CONDITIONS

Mg. acid / 100 ml.	Acid	Samp no	L	S	D	Hours since heating 1	3	5	7	11	15	21	23	47
6	DNA	1	x			.194		.247		.340			.570	
12	DNA	2-A		x		.234	.258	.276	.290		.325			.438
12	DNA	2-B		x		.236	.260	.280	.302		.340			.454
12	DNA	2-C		x		.233	.254	.273	.292		.330			.452
6	DNA	3			x	.193		.221		.220			.254	
	RNA	1	x			.408						.411		
	RNA	2	x			.412						.408		
	RNA	3	x			.422						.418		
	RNA	4	x			.407						.409		

L = color developed in light

S = on desk top in slotted wood test tube stands

D = color developed in dark

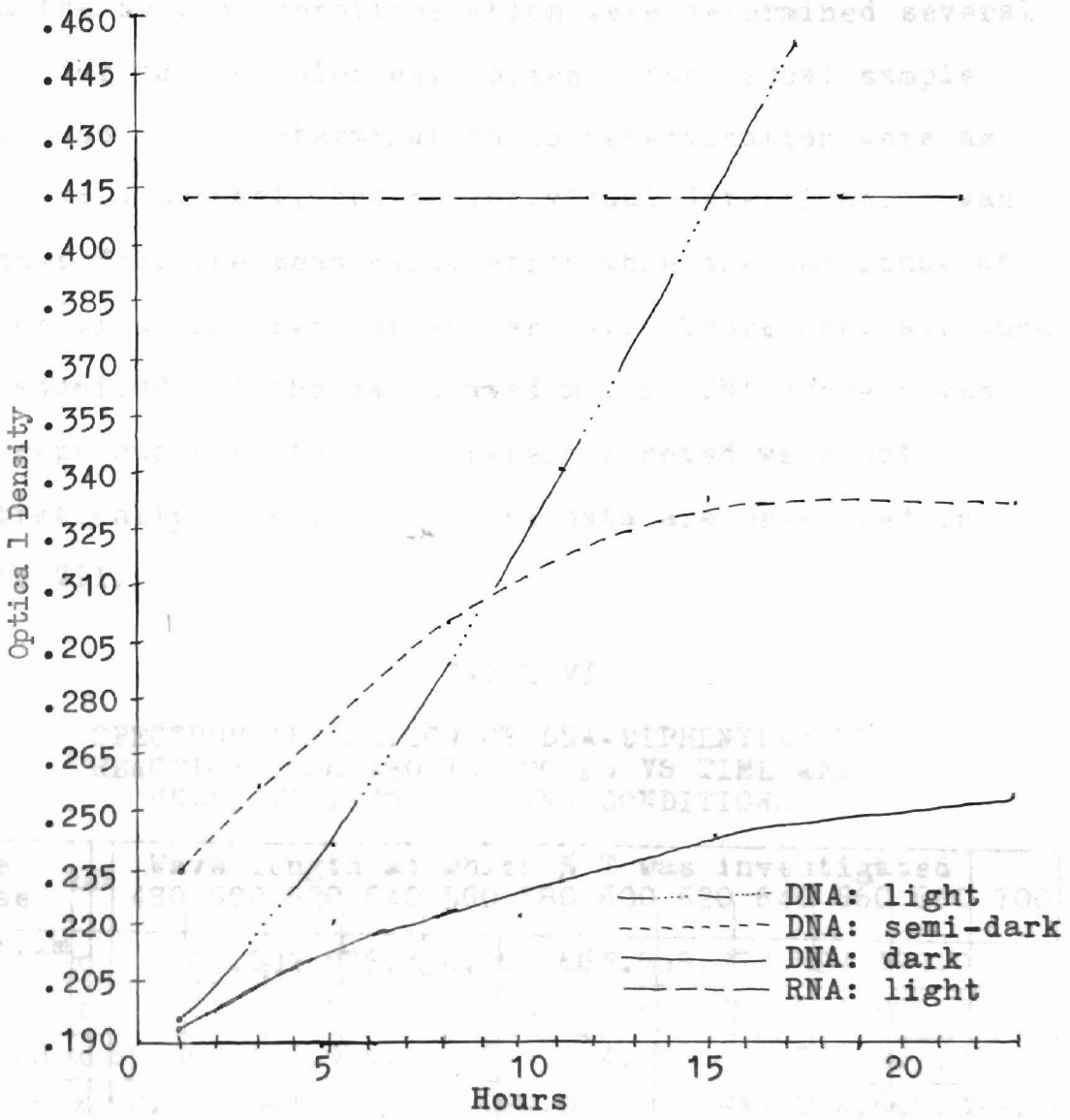

FIGURE 2

DNA AND RNA OPTICAL DENSITY CHANGE VS TIME
UNDER VARIOUS LIGHTING CONDITIONS

In calculating the mg. of DNA in a liver sample from the optical densities which were determined several times during the color development, individual sample variations from determination to determination were as large as 6 percent, but no individual determination was further from the mean calculation than the magnitude of the critical interval of 95 percent. Therefore, although the magnitude of the calculated mg. of DNA present was not reproducible, the differerences noted were not statistically significant. The data are presented in Table VII.

TABLE VI

SPECTRUM ABSORBTION OF DNA-DIPHENYLAMINE REACTION FROM 480 TO 700 mu VS TIME AND UNDER VARYING LIGHTING CONDITIONS

Time Lapse		L	D	Wave length at which % T was investigated 480	500	520	540	560	580	600	620	640	660	680	700
1 hr	12 m	x			84.0	82.0	76.3	70.8	65.6	63.8	68.2	77.5	87.1	93.0	
4	45	x			77.8	77.0	72.6	66.1	60.0	57.6	61.7	71.5	81.8	89.0	
11	48	x		65.8	62.1	60.9	56.1	50.0	45.5	44.2	48.8	58.7	69.2	77.0	80.9
23	38	x		50/3	48.0	44.5	38.5	32.0	28.0	27.2	31.9	42.2	54.6	65.0	71.2
1	25		x		85.0	82.9	78.2	72.0	66.4	64.2	68.5	77.4	87.0	93.3	
4	53		x		82.1	80.8	76.0	69.5	63.2	60.2	64.0	73.4	84.6	90.9	
11	58		x	85.0	84.8	82.9	78.0	70.8	64.0	60.9	64.7	74.1	84.5	92.0	
23	48		x	83.0	82.2	80.1	74.2	66.7	59.4	56.0	59.4	--	79.8	87.8	

L = color developed in light
D = color developed in dark

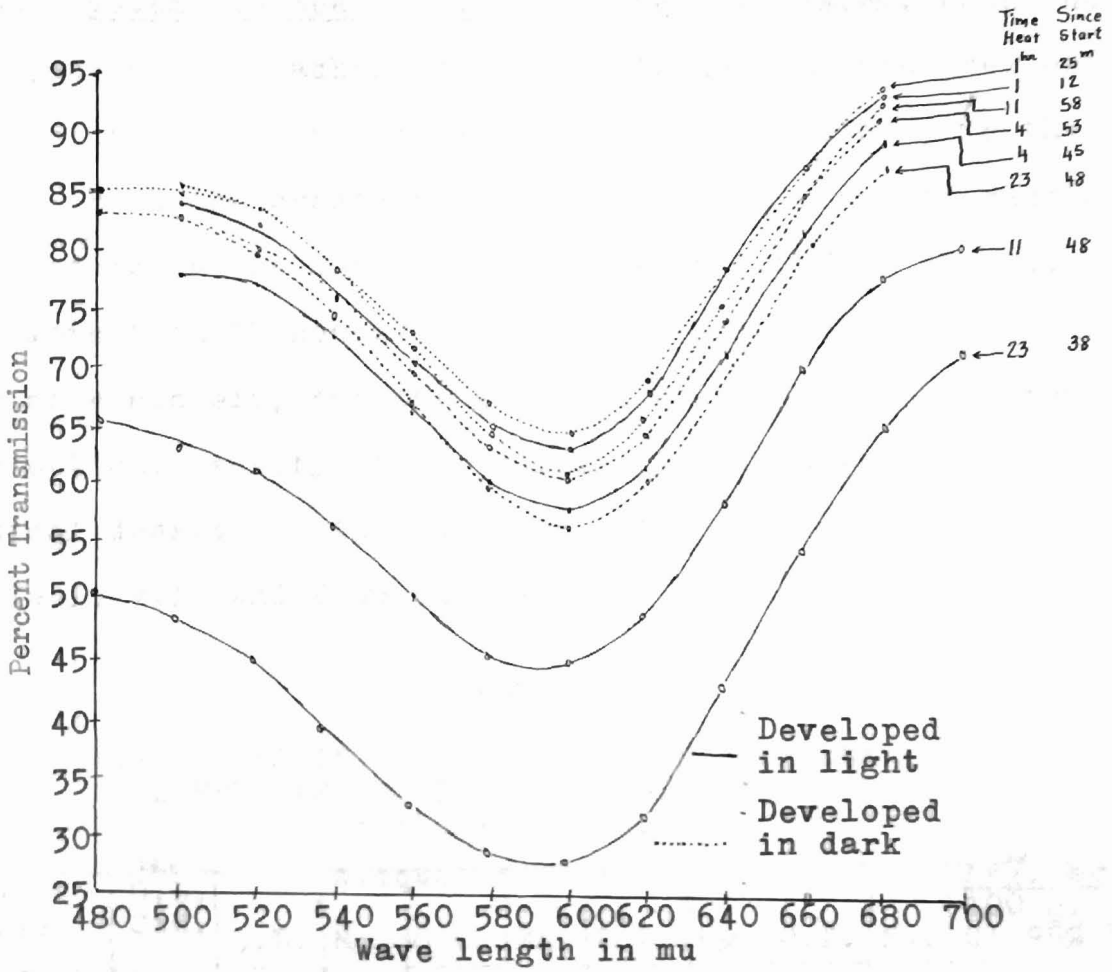

FIGURE 3

SPECTRUM ABSORBTION OF DNA-DIPHENYLAMINE REACTION OVER A PERIOD OF TIME AND UNDER VARYING LIGHTING CONDITIONS

Accuracy of DNA-RNA detection and the adherance of these systems to Beer's Law. In the DNA system, both the accuracy and the adherance to Beer's Law was the greatest for solutions which had had their color developed in the dark. Both the greatest accuracy and adherance to Beer's Law occured after the color had been allowed to develop for more than 15 hours. The RNA system adhered to Beer's Law more closely, but the greatest accuracy occured when the optical density of the sample closely approached the optical density of the standard. The data are presented in Table VIII and Figures 4 and 5.

TABLE VII

VARIATIONS IN CALCULATED AMOUNTS OF DNA IN LIVER SAMPLES DUE TO LENGTH OF COLOR DEVELOPMENT PERIOD

Time Lapse	O.D. Std.	Aliquot number 1		2		3		4		Avg. mg 100 g. CI±95%
		O.D.	Mg.	O.D.	Mg.	O.D.	Mg.	O.D.	Mg.	
2hr 10m	.246	.227	644	.217	614	.212	602	.236	668	632±61
5 20	.270	.248	640	.247	624	.232	598	.260	671	633±68
8 50	.298	.269	628	.262	613	.253	592	.282	668	625±72
12 22	.334	.308	643	.300	626	.387	598	.318	664	635±72
27 50	.355	.335	657	.319	626	.309	603	.349	685	643±78
Avg. mg. DNA/100 g CI±95%			642±20		621±9		601±8		674±15	

Concentration of standard: 12 mg. DNA/100 ml.
Mg. = Mg. DNA calculated per 100 g tissue

TABLE VIII

OPTICAL DENSITY AND ACCURACY OF DNA AND RNA SYSTEMS AS THEY VARY WITH DURATION OF COLOR DEVELOPMENT AND CONCENTRATIONS IN LIGHT AND DARK

Worker	Time Since Heat Start	Acid	Develop In Light	Develop In Dark	12 Mg ~ Known Amount		10 Mg ~ Known Amount		8 Mg ~ Known Amount		6 Mg ~ Known Amount		4 Mg ~ Known Amount	
					Optical Density	Mg Calculated	Optical Density	Mg Calculated	Optical Density	Mg Calculated	Optical Density	Mg Calculated	Optical Density	Mg Calculated
Garrigan	1 hr 54 min	DNA		X	0.200	Std	0.177	10.61	0.138		0.104	6.24		
"	1 34	"	X		0.247	"	0.188	9.14	0.152		0.112	5.44		
"	6 39	"		X	0.222	"	0.185	10.00	0.146		0.106	5.73		
"	6 49	"	X		0.340	"	0.235	9.00	0.185		0.145	5.13		
"	11 09	"		X	0.231	"	0.192	9.97	0.156		0.121	6.28		
"	10 40	"	X		0.359	"	0.258	8.63	0.198		0.155	5.18		
"	23 04	"		X	0.260	"	0.211	9.66	0.168		0.132	6.09		
Morrison	~24 hrs	"		X	0.396	"	0.316		0.210		0.168			
"	"	"		X	0.388	"	0.321		0.220		0.167			
"	"	"		X	0.398	"	0.315		0.210		0.170			
"	"	"		X	0.321	"	0.297	10.2 ± .4	0.240	7.53 ± .48	0.185	5.88 ± .19		
"	"	"		X	0.319	"	0.282		0.224		0.175			
"	"	"		X	0.320	"	0.285		0.230		0.173			
Garrigan	1 hr 12 min	"		X	0.221	"					0.115	6.24		
"	1 25	"	X		0.230	"					0.118	6.16		
"	4 45	"		X	0.243	"					0.130	6.42		
"	4 53	"	X		0.250	"					0.127	5.07		
"	11 48	"		X	0.248	"					0.129	6.23		
"	11 58	"	X		0.313	"					0.115	4.42		
"	23 38	"		X	0.283	"					0.147	6.24		
"	23 48	"	X		0.438	"					0.123	3.59		
"		RNA					0.585	9.69	0.483	Std	0.368	6.11	0.264	4.37
"		"			0.571	10.6	0.518	9.37	0.443	"	0.343	6.21	0.246	4.48

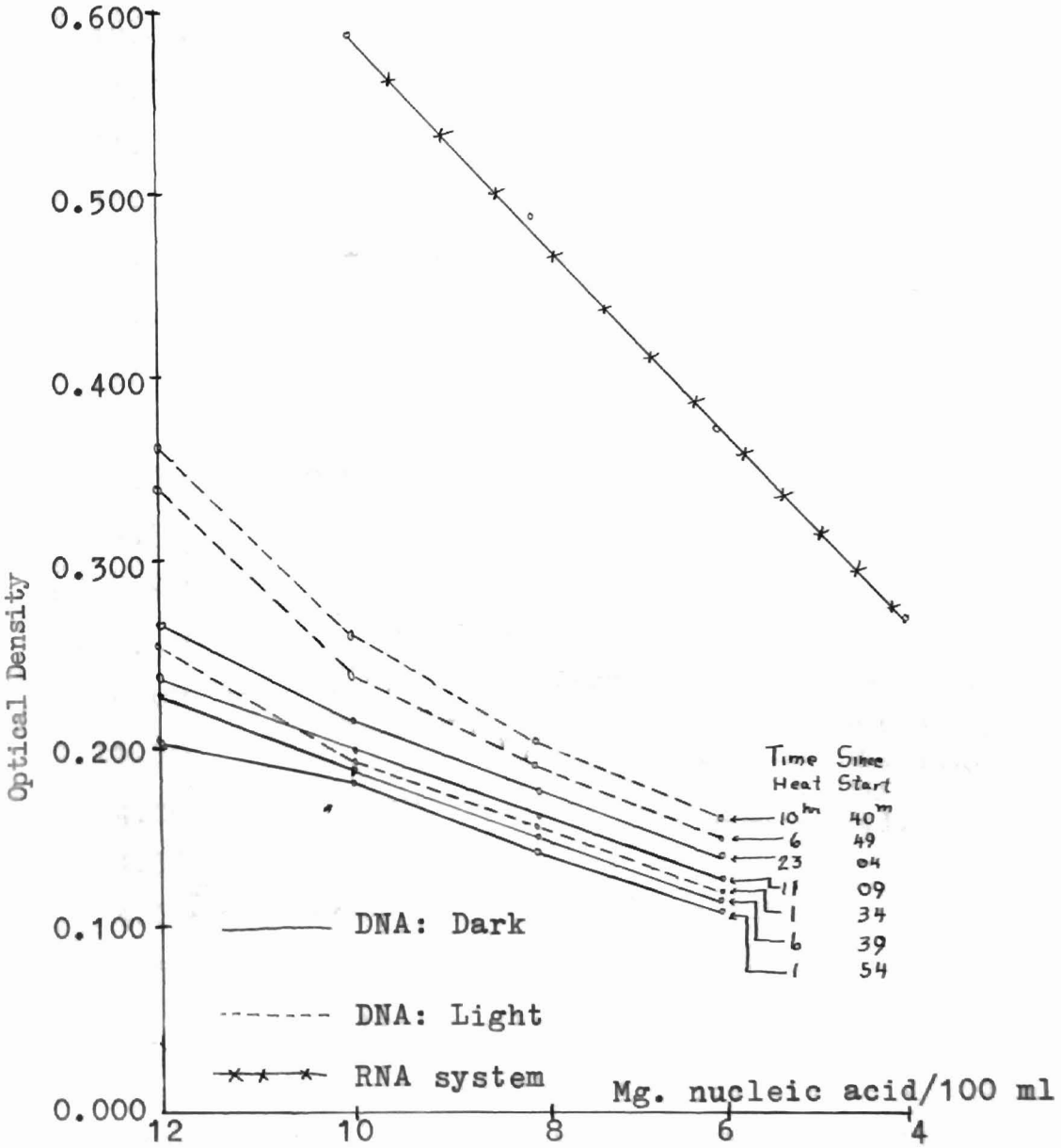

FIGURE 4

OPTICAL DENSITY OF RNA AND DNA SYSTEMS AS THEY VARY WITH DURATION OF COLOR DEVELOPMENT AND CONCENTRATION

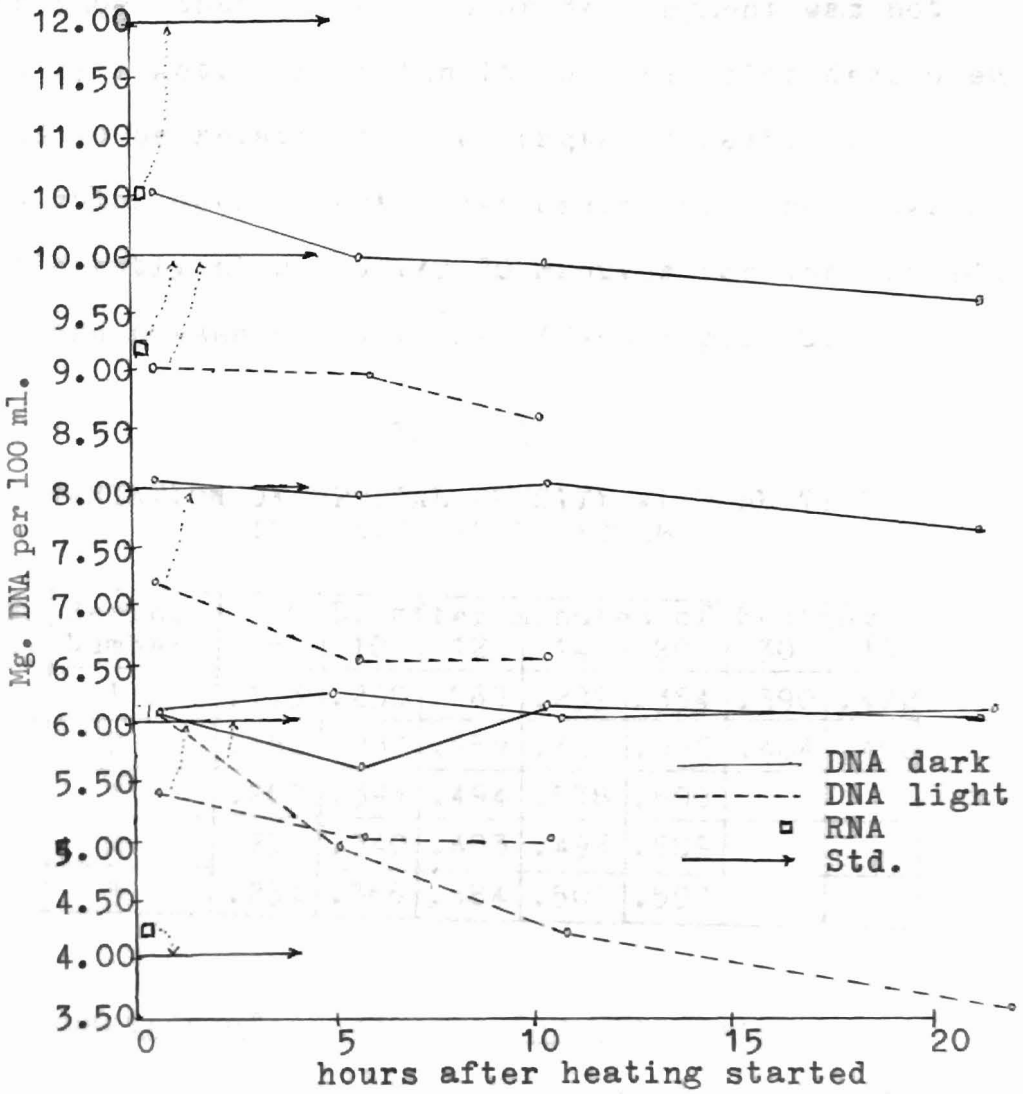

FIGURE 5

ACCURACY OF RNA-DNA SYSTEMS AS THEY VARY WITH DURATION OF COLOR DEVELOPMENT AND CONCENTRATIONS IN LIGHT AND DARK

RNA color development with heating time. Previous work had shown that the RNA color development was not affected by light. The intensity of the color developed was found to be related to the length of heating. Although color development never ceased with heating, it was sufficiently slowed after 20 minutes for the procedure. The data are presented in Table IX and Figure 6.

TABLE IX

VARIATION OF OPTICAL DENSITY WITH HEATING IN RNA-ORCINOL SYSTEM

Mg. RNA 100 ml.	Series Number	O.D. after minutes of heating 8	10	12	14	20	30	40
8	1	.175	.230	.269	.299	.354	.390	.422
8	2	.158	.237	.259	.309	.352	.404	.450
16	1	.269	.394	.494	.578	.598		
16	2	.255	.340	.483	.495	.589		
16	3	.254	.366	.484	.501	.597		

Comparison of procedures for phosphate determination. The comparison of tha acid and lipid soluble phosphate analyses procedures I and II showed that there was little difference in the reproducibility of either method and, therefore, neither is particularly better than the other. The data are presented in Table X.

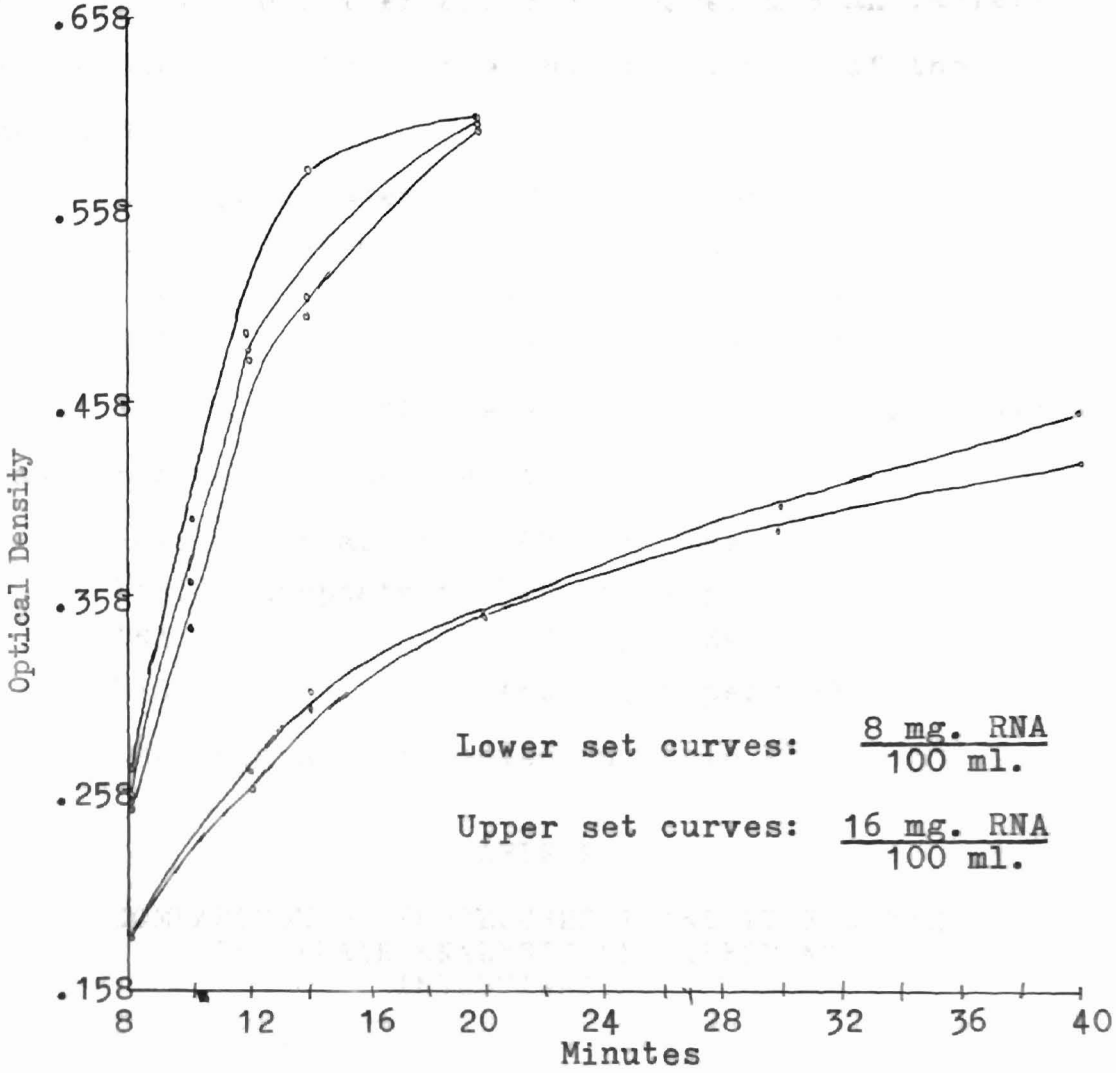

FIGURE 6

OPTICAL DENSITY OF STANDARD RNA
WITH LENGTH OF HEATING TIME

Analysis of procedure. The results of the analysis of the Cumberland mice showed a mean variation of the constituents of the individual mice of the magnitude:

Acid phosphates:	22 parts per 1000
Lipid phosphates:	28 parts per 1000
DNA:	100 parts per 1000
RNA:	60 parts per 1000.

The relationship between mice, however, showed a mean variation of the magnitudes:

Acid phosphates:	157 parts per 1000
Lipid phosphates:	380 parts per 1000
DNA:	630 parts per 1000
RNA:	450 parts per 1000.

The data are presented in Table XI

TABLE X

COMPARISON OF PROCEDURES I AND II FOR THE PHOSPHATE ANALYSIS BY DIFFERENT INVESTIGATORS

Investigator	Average % spread ((max-min)/mean)x100		Procedure used
	acid sol.	lipid sol.	
Carlson	7.5	13.8	II
Garrigan	4.0	6.4	I
Johns	7.0	12.1	II
Morrison	6.9	14.5	II
Spriggs	6.6	13.8	II
Suters	3.9	7.5	II

TABLE XI

ANALYSIS OF A-STRAIN, 8 TO 9 WEEK OLD, NORMAL, CUMBERLAND MICE

Mouse No.	Aliquot No.	Mg P/100 g tissue		Mg DNA / 100 g tissue	Mg RNA / 100 g tissue	Comments	Time of Death	RNA / DNA
		acid	lipid					
645	1	90.4	163	504	---	none	753	----
645	2	89.1	160	---	598	none	753	----
645	3	89.3	176	450	548	none	753	1.22
645	4	101.	162	484	593	none	753	1.22
646	1	94.1	150	576	747	none	1340	1.29
646	2	96.3	147	603	877	none	1340	1.45
646	3	94.2	149	551	774	none	1340	1.41
646	4	95.5	153	667	794	none	1340	1.19
647	1	100.	143	503	792	none	1913	1.57
647	2	101.	144	457	764	none	1913	1.67
647	3	102.	153	526	714	none	1913	1.36
647	4	101.	137	445	806	none	1913	1.81
648	1	88.6	125	232	738	mottled	930	3.19
648	2	91.6	131	241	734	mottled	930	3.05
648	3	89.1	128	247	---	mottled	930	----
648	4	86.9	133	210	662	mottled	930	3.15
649	1	107.	124	356	858	mottled	1340	2.42
649	2	112.	132	395	901	mottled	1340	2.28
649	3	106.	120	411	904	mottled	1340	2.20
649	4	105.	125	386	879	mottled	1340	2.28
650	1	98.5	146	445	822	none	1050	1.85
650	2	104.	143	473	834	none	1050	1.77
650	3	98.9	143	464	814	none	1050	1.75
651	1	90.4	121	418	860	none	1050	2.06
651	2	90.9	129	431	864	none	1050	2.01
651	3	90.5	129	394	847	none	1050	2.15
Mean: mottled livers excld.		95.1	146	434	773			1.63

Variations of constituents with age. Both the acid and lipid phosphates showed little or no variation with aging, the lipid phosphates being quantitatively greater and more prone to fluctuations. The nucleic acids showed considerable variability with the RNA being quantitatively greater than the DNA usually. No diurnal cycles of constituent variability was noted. The data are presented in Table XII and Figure 7.

TABLE XII

CONSTITUENT VARIABILITY OF NORMAL, A/JAX, FEMALE MICE WITH AGE

Date Death 1960	Mouse Number	Age in weeks	Sp Gr liver	Time of kill	Mg P/100 g tissue		Mg DNA / 100 g tissue	Mg RNA / 100 g tissue	RNA / DNA
					acid	lipid			
4/23	"6"	7	1.098	a.m.	94.7	133	---	---	----
6/27	577	8	1.050	1447	96.9	121	255	275	1.08
7/5	574	10	1.084	1515	105.	180	601	769	1.28
6/29	527	12	1.070	1845	113.	177	424	420	0.99
6/29	528	12	1.066	1340	115.	158	404	362	0.89
6/29	529	12	1.094	735	83.7	121	267	241	0.90
7/14	525	14	1.090	1325	105.	131	504	908	1.80
7/14	526	14	1.084	2025	100.	141	624	926	1.48
6/24	430	21	1.079	1445	97.3	183	322	527	1.64
7/20	468	22	1.100	755	111.	131	392	915	2.34
7/20	469	22	1.092	1300	99.0	134	390	844	2.16
7/20	470	22	1.087	1755	102.	147	466	874	1.87

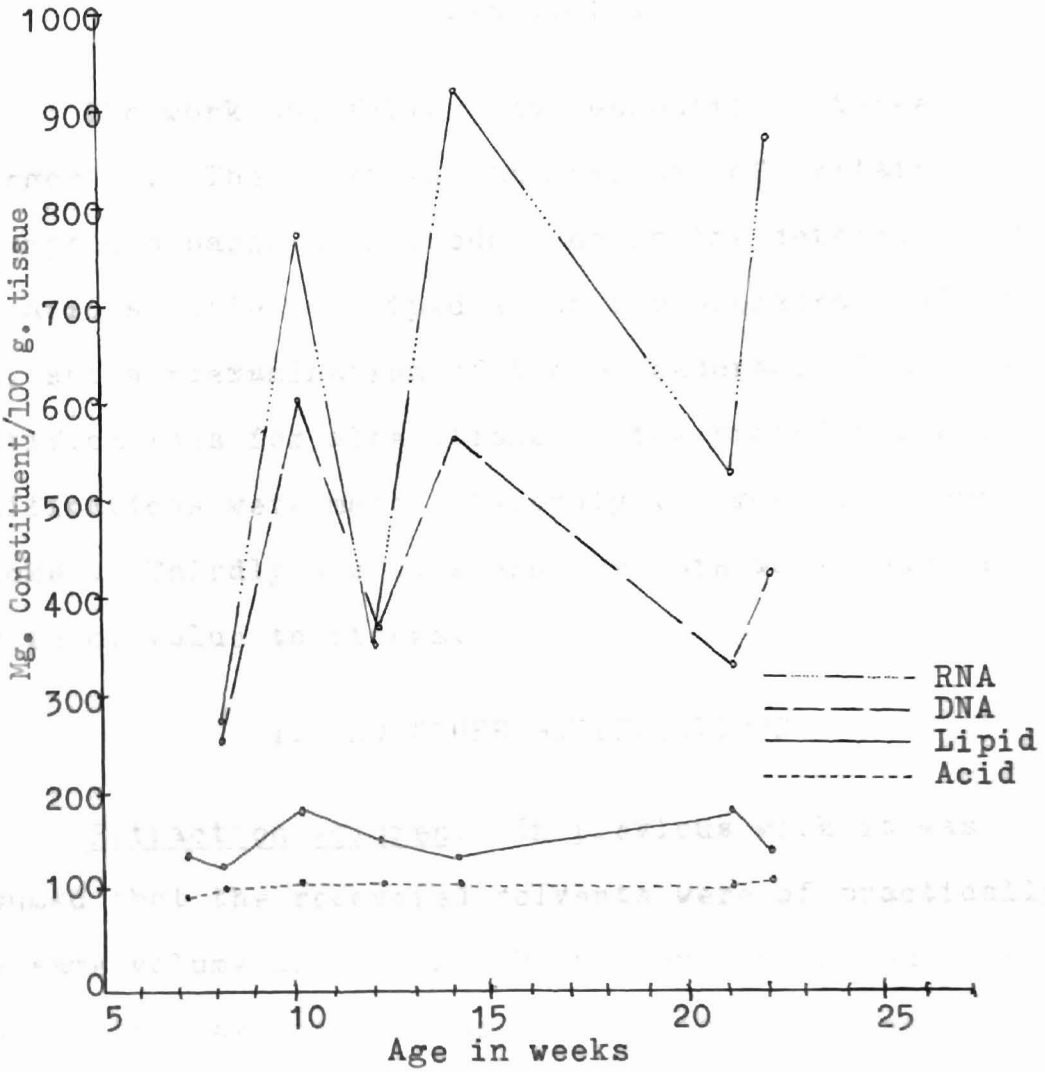

FIGURE 7

VARIATION OF CONSTITUENTS IN NORMAL,
A/JAX FEMALE MICE WITH AGE

CHAPTER IV

DISCUSSION

The work has fallen into essentially three categories. The first was an analysis of certain assumptions basic to methods used in the determination of the acid soluble and lipid soluble phosphates, DNA, and RNA, and a reexamination of the procedures. There were justifications for alterations in the procedure, and modifications were made. Secondly the modifications were checked. Thirdly new data and insights were found which may be of value to others.

I. PROCEDURE MODIFICATIONS

Extraction volumes. In previous work it was assumed that the recovered solvents were of practically the same volume as they had been when introduced. This was not true because the acid fraction consisted of solvent and the water of the homogenate, making the final volume greater than the original. A complete recovery of solvent from the lipid extraction was not possible because no sharp separation of solid and solvent occured during centrifugation. The final volume was, therefore, less than the original. The nucleic acid extract had a near

complete recovery of the solvent along with a recovery of some of the lipid solvent which had remained behind, and the final volume was usually greater than the original. As the final calculations depended on knowing the actual volume of the extract, there was need for the volume to be determined either through the use of tared test tubes or through dilution to a known volume. The former process was too laborious, and the latter was adopted.

Correction factor in RNA determination. Schneider noted that DNA gave a color with orcinol:

> "PNA and DNA gave E values per microgram of phosphorus of 0.135 and 0.0166 respectively in the orcinol reaction. The reaction of DNA with this reagent cannot be due to impurities in the DNA samples because all samples of DNA gave the same E values per microgram of phosphorus and because deoxyribose and the desoxyribossides also reacted with the orcino reagent.[1]

Dudley, using sperm from Nutritional Biochemical Corporation (lot number 5615), checked and obtained an optical density factor of 0.0156 per microgram of DNA.[2]

Both the author and Morrison checked Dudley's

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[1]W.C. Schneider, op. cit., LXXI (November 1945), p. 295.

[2]D.S. Dudley, et al., op. cit., pp. 428-429.

work and, using sperm from General Biochemical Inc (lot number 8044-C), obtained an optical density of 0.00405 per microgram of DNA.

The correction factor was of considerable importance in the determination of RNA andmore work should be done to determine if the discrepancies were due to sample impurities, sample decompositions, or some other cause.

DNA color development. The data quite conclusively shows that the color resulting from the diphenylamine-DNA reaction must develop in the dark. When allowed to develop in the light a deviation from the theoretical value of up to 41 percent was introduced after 24 hours of color development. During the same period, a solution maintained in the dark differed not over 5 percent from the theoretical concentration. The closest conformity to Beer's Law observed occured between 11 and 23 hours after heating was started. The spectral absorbtion pattern around 600 mu was not shifted when the color was developed either in the light or the dark.

RNA color development. The color resulting from the RNA-orcinol reagent reaction was stable in light. Apparently the color can develop only at high temperatures, but the rate of color development was

sufficiently slowed after 20 minutes heating to make it impractical to heat longer and inefficient to heat less. The reaction followed Beer's Law and the greatest accuracy occured when the standard-sample ratio approximated unity.

The acid and lipid soluble phosphates. Dudley reported discoloration after 10 minutes of digestion of the acid and lipid phosphates.[1] In the present study, discoloration occured after as much as 13 minutes after the addition of peroxide. Therefore a 15 minute digestion period was suggested.

The molybdenum blue developed much more rapidly in the light than in the dark, however the differences for the first 5 or 10 minutes were slight and the color could be allowed to develop in artificial light. However, the rate of color production was rapid enough to warrent accurately timing the length of development. The period of development should not vary more than one minute from sample to sample.

Minor Modifications. Minor modifications were introduced in concentrations and quantities used either

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[1]Mouse Liver Record Book II, p. 77.

for mechanical convenience or to adjust the optical density so that it would fall within the limits of minimal instrument error.

II. PROCEDURE ANALYSIS

In work with the 5 normal livers from the Cumberland mice, the relationship between this work and Dudley's of the mean quantities of the constituents was:

	Acid	Lipid	DNA	RNA	RNA/DNA
Dudley	94.2	155	319	1098	3.45
Garrigan	95.1	146	434	773	1.63

where values were in mg. of constituent per 100 g. of tissue.[1]

Variations could have been caused by a variety of factors. Dudley performed his study on A/Jax mice during the winter months while the present study was done on A-strain Cumberland mice during the summer using the modified procedure. Nevertheless, the work done on the Cumberland mice showed sufficient reproducibility in the writer's opinion to permit the usage of the procedure for the analysis of controlled mice.

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[1]D.S. Dudley, et al., op. cit., p. 429.

Analysis of A/Jax mice. Due to insufficient number of specimens available, little can be said specifically concerning the quantitative variations of the constituents of the normal liver with the aging of the mouse other than that both the acid and lipid phosphates showed little or no variation with aging, the lipid phosphates being quantitatively greater and more prone to fluctuation, and the nucleic acids showed considerable variability with the RNA being generally quantitatively greater than the DNA.

Other compounds and orcinol reagent. The orcinol reagent is used for the colorimetric detection of many compounds other than RNA.[1] Therefore, the fact that the acid and lipid fractions gave a positive reaction with the reagent neither positively indicated nor eliminated the possibility of the presence of RNA. On the other hand, there was no assurance that some of the color in the nucleic acid fraction was not due to the presence of some of these other compounds which may not have gone into one of the earlier solutions and which was not insoluble in the hot TCA.

Standard phosphate and hydronium ion. Hydronium

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[1]Snell and Snell, op. cit., p. 595.

ions from one source or another must be added to the standard phosphate solution before the heteropoly molybdiphosphoric acid (which is reduced to the molybdenum blue) can be formed.[1] If there was no such addition, the full color of the solution did not develop as was the case of solution I (page 9) in which the acid was not added in order to allow an investigation of the color development over a long period of time. Acid was automatically added during the analysis of tissues, but care had to be taken to see to it that it was added when investigations with the standard alone were undertaken.

Preparation of the nucleic acid standards. The mg. of nucleic acid per 100 ml. of the standard solution was calculated from the weight of the powdered standard which was used with no consideration given to the weight of the material, apparently inert, which did not dissolve in the KOH.

No investigation was undertaken to determine the rate of decomposition of the nucleic acid standards with time.

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[1] R.E. Kitson and M.G. Mellon, "Further Studies of the Molybdenum Blue Reaction," Industrial and Engineering Chemistry, Analytical Edition, XVI (1944), 466.

CHAPTER V

SUMMARY

In this study, special attention was given to the characteristics of colorimetric methods and reactions and to specific questions of technique involved in the procedure for the determination of the acid and lipid phosphates, DNA, and RNA in mouse hepatic tissues. Modifications in the procedure which were suggested were checked, and normal mice were analyzed with the modified procedure.

The study showed that the volume of the extract was not uniform and a dilution to a known volume was the most convenient way to obtain the needed known volumes. It also showed that the RNA correction factor was not the same as had been previously used, that the length of time for the molybdenum blue color development could not be allowed to vary more than one minute from sample to sample, that the acid and lipid phosphates gave a color with the orcinol reagent, that the color from the DNA-diphenylamine reaction had to develop in the dark, that the DNA absorbtion spectrum did not shift in the region from 480 to 700 mu when the color was developed either in the light or the dark, that the RNA-orcinol reaction had

to be heated 20 minutes and that the greatest accuracy in the RNA determination occured when the standard-sample ratio approximated unity. It further showed the degree of conformity of both the RNA and DNA solutions to Beer's Law, and the accuracy that can be expected in the determination of the nucleic acids with the procedure. Other small modifications in concentrations or volumes used were incorporated in the procedure for mechanical reasons.

The modified procedure was checked using 8 to 9 week old, normal, female mice from the Cumberland farms. Fourteen normal, age controlled, A/Jax female mice were analyzed and the variations of the acid and lipid soluble phosphates, RNA and DNA with the age of the mice were determined.

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LABORATORY NOTES

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